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13. ABSTRACT (Maximum 200 words)  The molecular basis for the anaerobic degradation of benzoate and 4-hydroxybenzoate was investigated using the bacterium <i>Rhodopseudomonas palustris</i> as a model. These aromatic acids are intermediates in the degradation of structurally diverse aromatic compounds, including environmental pollutants, by many metabolic types of anaerobic bacteria. Benzoate is the starting compound for a major central pathway of anaerobic benzene ring reduction and cleavage. This appears to be the major route required for complete degradation of toxic aromatic compounds under anaerobic conditions. There is still uncertainty, however, about the sequence of intermediates formed in the benzoate pathway and very little is known about the enzymology and genetics of the pathway. A major thrust of the work involved cloning benzoate degradation and regulatory genes and assigning functions to these genes based on nucleotide sequencing and physiological analyses of strains carrying defined mutations. Other studies included purification and characterization of the benzoate pathway ring cleavage enzyme (2-ketocyclohexanecarboxyl-CoA hydrolase).			
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**MOLECULAR BIOLOGY OF ANAEROBIC AROMATIC BIODEGRADATION**

**FINAL REPORT**

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**U. S. ARMY RESEARCH OFFICE**

**DAAL03-92-G-0313**

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#### A. STATEMENT OF THE PROBLEM STUDIED.

Chlorinated and nitro-aromatic compounds and aromatic hydrocarbons, including toluene and xylene, comprise a large proportion of the toxic wastes that have been released into the environment. Under anaerobic conditions the aromatic carboxylic acids, benzoate and 4-hydroxybenzoate, are formed as key intermediates during the biodegradation of these types of aromatic pollutants. The acids then enter central pathways of anaerobic benzene ring reduction and fission, leading to complete mineralization. Not a single catabolic pathway for the anaerobic degradation of any aromatic compound has yet been elucidated in detail, and practically no work has been done on the genes encoding pathway enzymes. If the potential of bacteria to degrade benzene rings under anaerobic conditions is to be manipulated to realize their full detoxification potential or to produce intermediary compounds that may have commercial value, it will be necessary to understand in detail the metabolic mechanisms involved, to know how the pathways are regulated, and to develop approaches for modifying the genes encoding key enzymes.

As an approach to achieving these goals we studied the anaerobic degradation of two selected aromatic acids - benzoate and 4-hydroxybenzoate - by one bacterial species - *Rhodopseudomonas palustris*. Our emphasis was on exploring the genetic basis of aromatic acid degradation. Our expectation is that it will be possible to extend many of our conclusions to other bacteria and related compounds.

#### B. SUMMARY OF THE MOST IMPORTANT RESULTS.

During the project period we completed sequencing and characterizing *aadR*, a gene which, based on sequence analysis, seemed likely to encode a transcriptional activator of anaerobic aromatic acid degradation genes. We also cloned, sequenced, and characterized the genes encoding benzoate and 4-hydroxybenzoate-CoA ligases; the enzymes that catalyze the initial reactions of benzoate and 4-hydroxybenzoate degradation by *R. palustris*. In addition, we constructed ligase mutants and characterized the mutant phenotypes. Finally, we carried out a physiological study of anaerobic cyclohex-1-ene-1-carboxylate metabolism. This alicyclic compound is a proposed intermediate of benzoate degradation. We identified cyclohex-1-ene-1-carboxylate degradation intermediates formed by benzoate-grown cells and also examined enzymatic activities involved in  $\beta$ -oxidation and cleavage of the alicyclic ring.

##### **1. We completed work on *aadR*, a gene which encodes a transcriptional regulator required for optimal expression of anaerobic 4-hydroxybenzoate and benzoate degradation genes. (Dispensa et al., 1992).**

The deduced amino acid sequence of the AadR protein indicates that it is a new and functionally distinct member of the family of transcriptional regulators which includes *E. coli* Crp and Fnr and *Rhizobium* FixK. An *aadR* deletion mutant failed to grow on 4-hydroxybenzoate and grew very slowly on benzoate. AadR mutants also failed to express 4-hydroxybenzoate-CoA ligase and were defective in 4-hydroxybenzoate-induced expression of benzoate-CoA ligase. The *aadR* mutation seems to specifically affect aromatic acid degradation. This is the first report of a regulator involved in controlling expression of genes for anaerobic aromatic compound degradation.

**2. We cloned and characterized the gene for 4-hydroxybenzoate-CoA ligase, the first enzyme of 4-hydroxybenzoate degradation (Gibson et al., 1994). We demonstrated that this gene is essential for growth on 4-hydroxybenzoate.**

This project was carried out in collaboration with Dr. Jane Gibson at Cornell University. Dr. Gibson purified and characterized the enzyme, and we cloned and sequenced the gene (*hbaA*) encoding the enzyme. We also constructed an *R. palustris* mutant carrying a disrupted *hbaA* gene and showed that it is incapable of anaerobic growth on 4-hydroxybenzoate, indicating that the *hbaA* product is an essential player in the 4-hydroxybenzoate degradation pathway. Anaerobic metabolism of diverse aromatic acid growth substrates is almost always initiated by coenzyme A (CoA) thioesterification, and several of these enzymes have been purified from two denitrifying *Pseudomonas* strains by Fuchs's group and also from *R. palustris* (we reported the purification of benzoate-CoA ligase in 1988). The characterization of 4-hydroxybenzoate-CoA ligase contributes to the development of a general picture of aromatic acid CoA ligases; the enzymes in this group have a number of properties in common, but also tend to have very specific and narrow substrate ranges. Our work is the first report of the cloning of a CoA ligase involved anaerobic degradation of aromatic compounds. The deduced amino acid sequence of 4-hydroxybenzoate-CoA ligase showed a low, but still significant, level of similarity to a number of fatty acid CoA ligase sequences (for example acetate-CoA ligase), as well as to the sequences of CoA ligases that participate in two somewhat unusual aerobic pathways of aromatic acid degradation. As more sequences of ligases involved in anaerobic aromatic degradation pathways become available, it should be possible to determine whether such enzymes fall into a distinct evolutionary subgrouping.

**3. We characterized the metabolites and enzymes of anaerobic cyclohex-1-ene-1-carboxylate ( $\Delta$ -1-chca) degradation by *R. palustris*. (Perrotta and Harwood, 1994) The CoA derivative of this alicyclic acid is a proposed intermediate of benzoate degradation and its conversion to pimelyl-CoA has been termed the  $\beta$ -oxidation phase of benzoate metabolism. We have partially purified the alicyclic ring cleaving enzyme.**

We synthesized appropriate substrates and developed refined assay conditions that have allowed us to detect the three  $\beta$ -oxidation activities involved in  $\Delta$ -1-chca-CoA degradation. Each of the enzymatic activities were typically five- to ten-fold higher in benzoate-grown than in succinate-grown cells. We also followed the degradation of [7-<sup>14</sup>C] cyclohex-1-ene-1-carboxylate by whole cells and determined that benzoate-grown cells formed metabolites that comigrated with cyclohex-1-ene-carboxyl-CoA, 2-hydroxycyclohexanecarboxyl-CoA, 2-ketocyclohexanecarboxyl-CoA, and pimelyl-CoA by thin-layer chromatography. Our results have provided a first demonstration of an alicyclic ring cleavage activity (the 2-ketocyclohexanecarboxyl-CoA hydrolase). We partially purified this enzyme and did an initial characterization of its properties.

**4. We cloned and sequenced the gene, *badA*, encoding benzoate-CoA ligase. We demonstrated that this gene is not essential for growth on benzoate, indicating that benzoate-grown *R. palustris* cells synthesize more than one enzyme able to catalyze benzoyl-CoA formation.**

The benzoate-CoA ligase gene is 1,565 bp in length and has about 45% sequence identity at the amino acid level to the *hbaA* gene encoding 4-hydroxybenzoate-CoA ligase. The proteins encoded by these genes have very distinct properties and antisera prepared against each enzyme do not cross-react. Although enzymological data had indicated that the *badA* product is the principle enzyme involved in initiating anaerobic benzoate degradation in *R. palustris*, this organism also has two other enzymes, a 4-hydroxybenzoate-CoA ligase and a cyclohexanecarboxylate-CoA ligase that are active with benzoate. Using a combination of genetic and physiological approaches we demonstrated that all three of these enzymes can catalyze the initial step of anaerobic benzoate degradation during growth on benzoate. Thus *R. palustris* has two "back-up" systems to ensure the initial activation of benzoate.

#### C. LIST OF PUBLICATIONS.

##### Journal articles:

- Dispensa, M., C. T. Thomas, M.-K. Kim, J. A. Perrotta, J. Gibson and C. S. Harwood. 1992. Anaerobic growth of *Rhodopseudomonas palustris* on 4-hydroxybenzoate is dependent on AadR, a member of the cyclic AMP receptor protein family of transcriptional regulators. *J. Bacteriol.* 174:5803-5813.
- Gibson, J., M. Dispensa, G. C. Fogg, D. T. Evans, and C. S. Harwood. 1994. 4-Hydroxybenzoate-coenzyme A ligase from *Rhodopseudomonas palustris*: purification, gene sequence, and role in anaerobic degradation. *J. Bacteriol.* 176:634-641.
- Perrotta, J. A. and C. S. Harwood. 1994. Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation by *Rhodopseudomonas palustris*. *Appl. Environ. Microbiol.* 60:1775-1782.

##### Published abstracts:

- Perrotta, J. A. and C. S. Harwood. 1993. Cyclohex-1-enecarboxyl-CoA conversion: the " $\beta$ -oxidation" phase of anaerobic benzoate degradation by *Rhodopseudomonas palustris*. *Abstr. Ann. Meet. Amer. Soc. Microbiol.* 93:Q60.
- Egland, P.G., and C. S. Harwood. 1994. Anaerobic aromatic compound degradation: cloning of the benzoate-coenzyme A ligase gene from *Rhodopseudomonas palustris*. *Abstr. Ann. Meet. Amer. Soc. Microbiol.* 94:Q412.
- Egland, P. G., and C. S. Harwood. 1995. Contribution of *badA*, encoding benzoate-coenzyme A ligase, to the anaerobic degradation of benzoate by *Rhodopseudomonas palustris*. *Abstr. Ann. Meet. Amer. Soc. Microbiol.* 95:Q320.
- Pelletier, D. A. and C. S. Harwood. 1995. Purification of 2-ketocyclohexanecarboxyl-coenzyme A hydrolase, a ring-cleavage enzyme from anaerobic benzoate-grown *Rhodopseudomonas palustris*. *Abstr. An. Meet. Amer. Soc. Microbiol.* 95:Q322.

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D. SCIENTIFIC PERSONNEL SUPPORTED BY THIS PROJECT AND DEGREES AWARDED.

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Joseph A. Perrotta (Graduate Research Assistant) - M.S. awarded

REPORT OF INVENTIONS:

None